Award Number: DAMD17-01-1-0793

TITLE: Disruption of Dopaminergic and Cholinergic Function in Military Deployment Implications to Parkinson's Disease

PRINCIPAL INVESTIGATOR: Gary W. Miller, Ph.D.

CONTRACTING ORGANIZATION: Emory University
Atlanta, Georgia 30322

REPORT DATE: November 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	November 2004	Annual (15 Aug 2003 - 14 Aug 2004)

4. TITLE AND SUBTITLE
Disruption of Dopaminergic and Cholinergic Function in Military Deployment Implications to Parkinson's Disease

5. FUNDING NUMBERS
DAMD17-01-1-0793

6. AUTHOR(S)
Gary W. Miller, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Emory University
Atlanta, Georgia 30322

E-Mail: gwmille@emory.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The goal of this project is to evaluate the potential of pesticides and other compounds used by the military for their potential to damage the brain dopamine system and increase the risk for Parkinson's disease. Notable research accomplishments over the past year include the following: Deltamethrin increased the expression of DAT, TH, and VMAT2, locomotor activity in C57BL mice. Deltamethrin did not exacerbate the toxicity to the dopamine neurotoxin MPTP either given before or after MPTP. Chlorpyrifos had no effect on dopamine uptake in neuroblastoma cells and did not exacerbate MPTP toxicity. Pyridostigmine bromide had no effect on dopamine uptake in neuroblastoma cells. JP-8 jet fuel is toxic to neuroblastoma cells only at 1 mM concentrations. No toxicity was seen at concentrations from 100 nM to 500 μM. Thus, with all of the compounds studied we have not observed toxicity consistent with a compound that would be thought to cause overt damage to the dopamine system. However, we have seen alterations of the dopamine system that must be studied further. The completion of this study will reveal the impact of militarily relevant agents on the pathogenesis of Parkinson's disease and hopefully lead to strategies and policies that reduce the incidence of the disease.

14. SUBJECT TERMS
Pyrethroid, acetylocholinesterase inhibition, deltamethrin, pyridostigmine bromide, microarrays

15. NUMBER OF PAGES
35

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

This report summarizes the key findings of Year 3 of this project. The following Statement of Work is excerpted from the original project, but has been modified to include the supplement on JP-8 jet fuel. Dr. Miller has established a collaborative relationship with Dr. Jeff Fischer at the University of Georgia that should benefit this project. Dr. Fischer studied pharmacokinetics of JP8 and related compounds at Wright Patterson Airforce Base for 20 years before moving to UGA. While we only proposed to do intraperitoneal injections for our Statement of Work, Dr. Fischer has agreed to let us harvest brain tissue from animals exposed to JP8 vapor. We think this will add significant value to our studies by allowing us to compare the i.p. exposure to the more relevant inhalation exposure. Dr. Fischer has also suggested that we try some dermal exposures and we are currently exploring this possibility.

Specific Aim 1. Effects of pyrethroids, acetylcholinesterase inhibitors, and JP-8 jet fuel on dopamine uptake, DAT localization, and MPP^+ induced apoptosis in DAT expressing cells. This aim will test the hypothesis that pyrethroids and acetylcholinesterase inhibitors increase MPP^+-induced apoptosis primarily through acting on dopamine uptake. The following experiments will be performed under this aim:

I) Perform dopamine uptake tests on DAT expressing cells treated with deltamethrin, permethrin, chlorpyrifos, pyridostigmine bromide, and MPP^+. Years 1-2

II) Determine the effects of deltamethrin, permethrin, chlorpyrifos, pyridostigmine bromide, JP-8, and MPP^+ on DAT localization in DAT expressing cells. Year 3-4

III) Perform caspase 3 assays on cells treated with deltamethrin, permethrin, chlorpyrifos, JP-8, and pyridostigmine bromide to determine if they cause apoptosis or exacerbate MPP^+-mediated apoptosis. Year 1-2

Specific Aim 2. Examine the effects of pyrethroids, acetylcholinesterase inhibitors, JP-8, and MPTP on mouse behavior and dopaminergic and cholinergic gene and protein expression. This aim will test the hypothesis that the combination of pyrethroids and acetylcholinesterase inhibitors decreases dopaminergic activity and increases cholinergic activity, resulting in impaired locomotion in C57BL/6 mice. *An important feature of this aim is that we will examine behavior, gene expression, protein expression, and neurotransmitter levels in the same animals.*

Aim 2A. Assess effects of pyrethroids, acetylcholinesterase inhibitors, and their combination on mouse behavior. In addition, JP-8 will also be tested. This aim will test the hypothesis that these compounds decrease locomotion and increase anxiety and aggression.

IV) Perform locomotor activity, open field ambulation, elevated plus maze, and social interaction tests on C57BL mice six days after MPTP treatment. 3 days prior to or 3 days following MPTP treatment, mice will be treated with 9 mg/kg of deltamethrin, chlorpyrifos, neostigmine, or the combination of deltamethrin and chlorpyrifos. Year 1, 2

Aim 2B. Immunochemical and neurochemical analysis of dopaminergic and cholinergic systems following pyrethroids, acetylcholinesterase inhibitors, JP-8, and MPTP. This aim will assess the effects of pyrethroids, acetylcholinesterase inhibitors, and MPTP on cholinergic and dopaminergic protein expression and function.

V) On the same mice in Aim 2A perform immunoblotting for DAT, D1, tyrosine hydroxylase, M1 and M2 receptors, vesicular acetylcholine transporter, and choline acetyltransferase. Year 1.5 to 2.5

VI) Perform HPLC analysis of monoamines on mice from Aim 2A. Year 2

VII) On a separate subset of animals treated with deltamethrin, chlorpyrifos, neostigmine, and MPTP, perform striatal dopamine and choline uptake. Year 3.

Aim 2C. Use custom cDNA microarrays to analyze regional changes in dopaminergic and cholinergic gene expression following pyrethroids, acetylcholinesterase inhibitors, JP-8, and MPTP. VIII) We will perform cDNA microarray analysis on midbrain, basal forebrain, and striatum from ice treated with deltamethrin, chlorpyrifos, or MPTP. Years 2 and 3 will contain most of the actual hybridizations. Year 4 will be focused bioinformatic analysis. Years 2,3,4

Scientific Progress

We have made significant progress in our Statement of Work. As reported in the progress report from the previous year. We have finished the experiments in Specific Aim 1 with the pyrethroids (deltamethrin and permethrin) and cholinesterase inhibitors (chlorpyrifos-oxon and pyridostigmine bromide) and have identified that none of these compounds are capable of directly affecting dopamine transporter (DAT) function in cells stably expressing the human DAT. However, prolonged exposure to the pyrethroid resulted in decreased dopamine uptake. We demonstrated that this effect was not the result of overt toxicity, but coincided with the appearance of DNA fragmentation, indicative of an ongoing apoptotic process. These experiments are now in manuscript form and will be submitted to Toxicology and Applied Pharmacology by the end of the calendar year (*see appendix*). In the last report, we provided dose-response data on the effects of JP-8 jet fuel on cytotoxicity in SK-N-MC neuroblastoma cells. We are currently in the process of performing the apoptosis and dopamine uptake assays proposed in Specific Aim 1. While SK-N-MC cells have been the standard cell line we have used in our laboratory, they are primarily cholinergic. We have recently obtained a dopaminergic cell line (N27) isolated from fetal rat mesencephalic neurons and transformed with SV40. We feel that these cells will be ideal for looking at relative susceptibility of dopaminergic cells to the compounds proposed in our Statement of Work. We will continue to perform assays with the SK-N-MC cells as well.

In Specific Aim 2, we proposed to examine the effects of pyrethroids, cholinesterase inhibitors, and their combinations on mouse behavior and dopaminergic and cholinergic gene and protein expression. Much of this work, including the effect of pre- or post-exposure to these compounds on MPTP toxicity was reported last year. As reported, the single exposures to pyrethroids or cholinesterase inhibitors had no effect on the DAT. We provided preliminary evidence that repeated lower-level exposure to deltamethrin resulted in increased DAT levels. We have extended those observations to include studies with permethrin. We have found that administration of deltamethrin (3 mg/kg) or permethrin (0.8 mg/kg) three

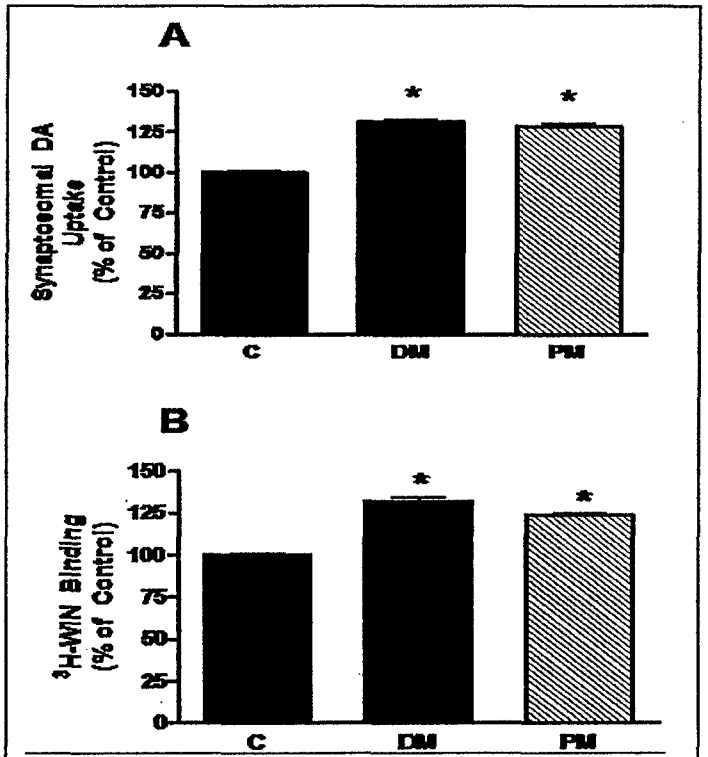


Figure 1. Exposure to deltamethrin (DM) or permethrin (PM) increases DAT-mediated dopamine uptake and ^{3}H-WIN 35,428 binding to DAT in striatal synaptosomes. Mice were administered DM (3 mg/kg) or PM (0.8 mg/kg) 3 times over a 2 week period and sacrificed 1 day after the last exposure.

times over a two week period to C57BL/6j mice results in a significant increase in DAT-mediated dopamine uptake in striatal synaptosomes prepared from these mice (Figure 1A). This up-regulation of DAT function was accompanied by an increase in the number of DAT binding sites as measured by ^{3}H-WIN 35,428 binding to DAT in synaptosomes prepared from these mice. *This is a particularly important finding since military personnel are most likely to be exposed to low-levels of these compounds over a period of time rather than a single large dose.* The dose of deltamethrin administered in these studies (3 mg/kg) is 3-fold lower than the 9 mg/kg used in the single exposure studies reported last year. Likewise, the dose of permethrin (0.8 mg/kg) is 11-fold lower than the 9 mg/kg used in the single exposure studies. Mechanistically, the studies performed in Specific Aim 1 suggest that the effects observed with the *in vivo* exposures are not the result of a direct effect on DAT itself.

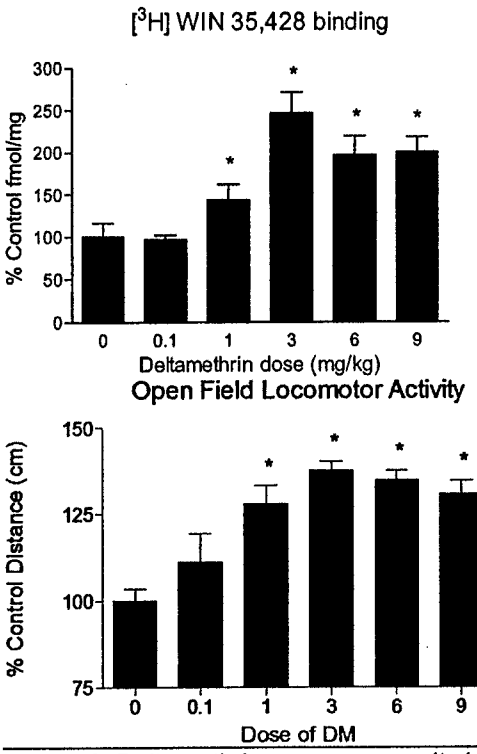


Figure 2. Deltamethrin exposure results in a dose-related increase in DAT levels and locomotor activity. C57BL/6j mice were treated with various dosages of DM 3 times over a 2 week period. Locomotor and DAT determinations were made 1 day following the last injection.

We have extended these studies further with deltamethrin and determined a dose-response relationship between deltamethrin exposure and up-regulation of DAT. As can be seen in Figure 2 (Top), 1 mg/kg administered every three days over 2 weeks appears to be the lowest dosage capable of increases DAT levels. We also determined that the up-regulation of DAT was accompanied by increased locomotor activity in the open-field (Figure 2 Bottom). Indeed, we found a significant correlation between the levels of DAT, as determined by WIN binding, and increased locomotor activity (Figure 3). Therefore, it appears that the functional up-regulation of DAT by deltamethrin exposure has functional consequences on the behavior of the animal that is manifested as hyperactivity. We have also performed preliminary experiments with

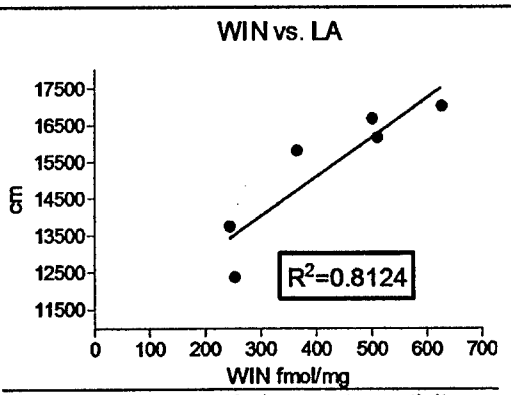


Figure 3. Increases in locomotor activity Following deltamethrin exposure correlate Well with the increases in DAT levels.

dopamine receptor antagonists and found that they were able to attenuate the hyperactivity. These data suggest that the hyperactivity is dopaminergic in origin.

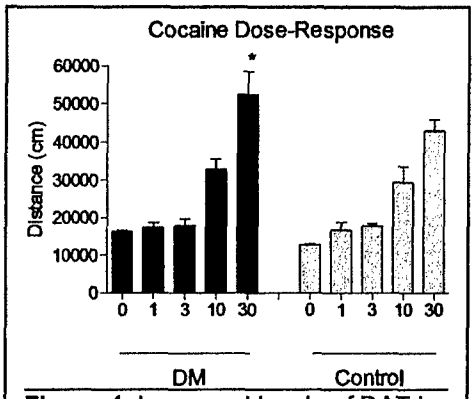


Figure 4. Increased levels of DAT in deltamethrin treated mice result in enhanced locomotor response to cocaine.

In addition to the hyperactivity noted in these mice, we have determined that mice exposed to deltamethrin display increased response to cocaine-induced locomotion (Figure 4). While not part of the original application, these data provide further mechanistic insight into the consequences of low-level pyrethroid exposure. These data also suggest that up-regulation of DAT by pyrethroid exposure may exacerbate responses to therapeutic drugs which work through DAT, such as the anti-depressant and smoking-cessation aid bupropion (Wellbutrin).

In Aim 2, we proposed to determine whether pyrethroid or cholinesterase inhibiting pesticides increased anxiety. Figure 6 demonstrates that mice exposed to 3 mg/kg of deltamethrin every three days for two weeks have decreased anxiety as evidenced by increased time spent in the center of the open-field. Generally, mice fear open space and the center of the open field. The top part of the graph is a track-trace of mouse movement in an open-field for 90 minutes following a 30 minute acclimatization period. As can be seen in the graph, control mice spent the majority of their time in the peripheral part of the open-field box away from the center. However, deltamethrin treated mice were hyperactive and spent more time crossing the open-field and in the center (Figure 5). Whether these results are mainly because of the hyperactivity induced by deltamethrin or truly indicate decreased anxiety remains to be established. Therefore, we will assess anxiety related behaviors in the elevated plus maze to determine the role of hyperactivity in this behavior. These studies are ongoing.

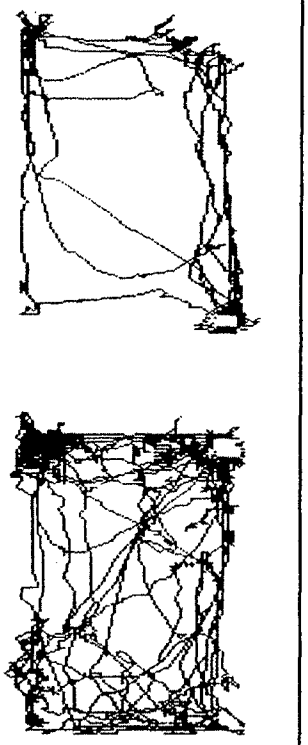


Figure 5. Deltamethrin treated mice display increased anxiety as determined by increased time spent in the center of the open field. Top. Control mouse. Bottom. Mouse administered 3 mg/kg deltamethrin.

Concurrently with our studies on DAT in mice treated with pyrethroids, we have isolated RNA from these mice for use with real-time PCR profiling of genes involved in dopaminergic and cholinergic function. We currently have real-time probes developed for DAT, VMAT2, tyrosine hydroxylase, D1-D5 dopamine receptors, DARPP-32, choline acetyltransferase, high affinity choline transporter,

M1-M5 muscarinic receptors, acetylcholinesterase, and butyrylcholinesterase. We have also assessed the effects of deltamethrin exposure on other monoaminergic systems. We found that the effects of deltamethrin appear to be preferential for DAT, as cortical levels of the serotonin and norepinephrine transporter were not affected by *in vivo* exposure to deltamethrin (Figure 6). We have taken tissue from these animals to assess the effects of pyrethroids on cholinergic neurochemistry as well. As reported in last year's progress report, single administration of chlorpyrifos or pyridostigmine bromide had no effects on dopaminergic or cholinergic neurochemistry. Therefore, we are proposing to perform similar repeated dosing as we have with the pyrethroids with chlorpyrifos and pyridostigmine bromide.

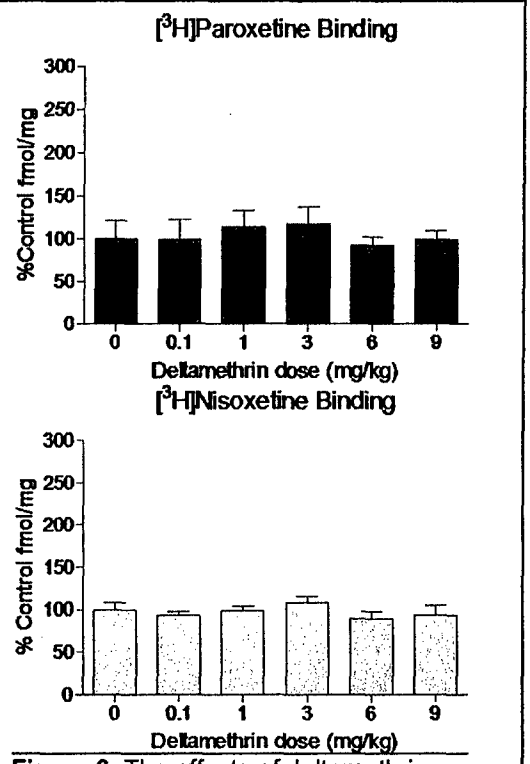


Figure 6. The effects of deltamethrin appear to be preferential for the dopamine system, as there were no effects on cortical levels of the serotonin transporter (paroxetine binding) or norepinephrine transporter (nisoxetine binding).

As outlined in last year's progress report, we found no differences in locomotor activity in animals treated with MPTP or the combination of MPTP and the pesticides in this proposal. This is consistent with the findings of our lab that many of these routine tests are not at all sensitive to motor impairment in rodent models of neurodegeneration. To provide a more sensitive measure of toxin-induced behavioral deficits, we have recently installed a sophisticated gait-analysis system from Mouse Specifics, Inc. The mouse walks on a transparent conveyor belt and the paw placement is captured digitally from below with a video camera. Patterns of gait dynamics for each limb of the walking rodent are analyzed for stride length, stance width, stance time, braking time, and propulsion time – indices that may demonstrate the effects of genes and/or drugs on coordinated movement. We have validated this test using a moderate dosage (2x10 mg/kg) of MPTP. This dosage produces approximately a 50%

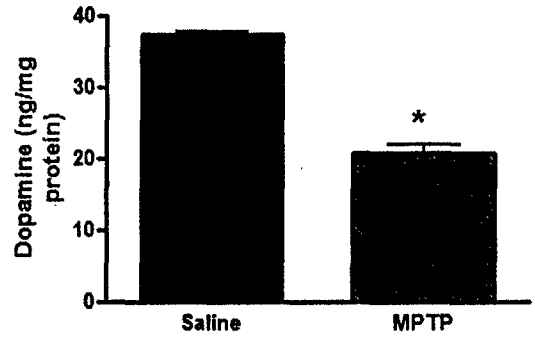


Figure 7. Striatal dopamine levels are decreased by 50% in mice administered 2 x 10 mg/kg MPTP twice over a 12 hr period. This is a very moderate dose and lesion compared to most MPTP studies which routinely deplete over 90% of striatal dopamine.

loss in striatal dopamine following treatment (Figure 7). At this level, no currently available behavioral tests can readily detect behavioral deficits. Using the new Cleversys software for the gait analysis apparatus, we systematically analyzed over 15 individual measurements related to the gait of the mouse. We found that a moderate MPTP lesion produced significant increases in forepaw brake time, while decreasing hindpaw brake time (Figure 8). We also found alterations in forepaw and hindpaw stride length (Figure 9). These data suggest that the automated gait analysis system is a sensitive and detailed method for detecting behavioral deficits in mice with moderate dopaminergic lesions. We are currently in the process of applying these tests to the pyrethroid treated animals.

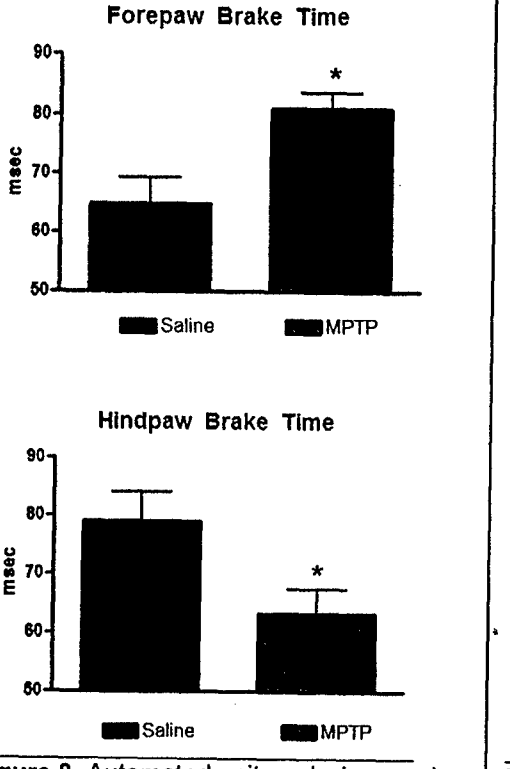


Figure 8. Automated gait analysis reveals increased forepaw and decreasedhindpaw brake time in mice administered a moderate dose of MPTP.

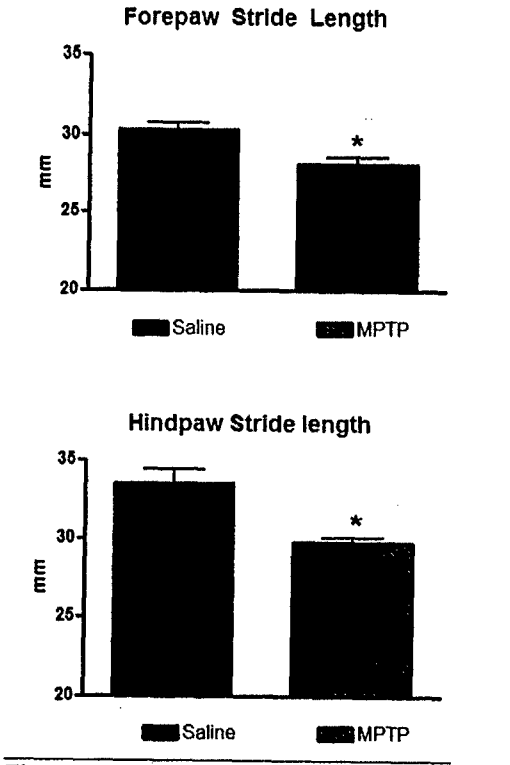


Figure 9. Automated gait analysis reveals decreased forepaw and hindpaw stride length in mice administered a moderate dose of MPTP.

References
None

Appendix
Copy of manuscript to be submitted to Toxicology and Applied Pharmacology before the end of the calendar year
Copy of poster presented at the Society of Toxicology Annual Meeting
Copy of poster presented at the Society of Neuroscience Annual Meeting
Copy of abstract submitted for presentation at Society of Toxicology Annual Meeting

Key Research Accomplishments for Year 3
Repeated exposure to deltamethrin increases dopamine uptake and expression of the dopamine transporter in C57BL/6j mice
Repeated exposure to permethrin increases dopamine uptake and expression of the dopamine transporter in C57BL/6j mice
Repeated exposure to deltamethrin does not affect serotonin or norepinephrine transporter levels in C57Bl/6j mice
Repeated exposure to deltamethrin causes hyperactivity which is correlated to increased dopamine transporter expression in C57BL/6j mice
Repeated exposure to deltamethrin increases cocaine-induced locomotor activity
Repeated exposure to deltamethrin increases anxiety as assessed by increased center time in the open field
Development of methodology for sensitive-automated gait analysis test to detect behavioral impairment in moderately-lesioned MPTP mice

Reportable outcomes:
Poster presented at SOT meeting

Guillot, T.S., Richardson, J.R., and Miller, G.W. (2004). Deltamethrin Increases Dopamine Transporter Expression and Enhances Cocaine-induced Locomotion. Toxicologist 78:1357.

Poster presented at Society of Neuroscience Meeting

Guillot, T.S., Richardson, J.R., and Miller, G.W. (2004). Pesticide Exposure Upregulates the Dopamine Transporter and Increases Cocaine-induced Locomotor Activity while Abolishing Place Preference. Society for Neuroscience Abstracts 804.14.

Abstract submitted for presentation at SOT Meeting

Guillot, T.S., Richardson, J.R., and Miller, G.W. (2005). Sensitive Detection of Behavioral Impairments in Moderately Lesioned MPTP Mice by Automated Gait Analysis. Submitted for SOT Annual Meeting.

Manuscript to be submitted for publication in Toxicology and Applied Pharmacology

Elwan, M.A., Richardson, J.R., Guillot, T.S., Caudle, W.M., and Miller, G.W.

Deltamethrin Increases Dopamine Transporter Expression and Increases Basal and Cocaine-Induced Locomotion

Thomas S. Guillot, Jason R. Richardson and Gary W. Miller

Center for Neurodegenerative Disease and Department of Environmental and Occupational Health, Rollins School of Public Health, Emory University, Atlanta, GA.

ABSTRACT

Deltamethrin (DM), a type II pyrethroid insecticide, was introduced in the early 1980s and has become one of the most widely used insecticides in both agricultural and household settings. Recently, pyrethroid exposure has been shown to preferentially affect nigrostriatal dopaminergic neurons. In particular, a functional upregulation of the dopamine transporter (DAT) has been demonstrated in the striatum of C57BL/6 mice exposed to DM (Kirby et al. 1999). DAT, in addition to its normal role in dopamine clearance, is a target for psychostimulants such as cocaine. In fact, mice in which DAT was genetically overexpressed by 20-30% exhibited increased locomotor activity in response to cocaine challenge (Donovan et al. 1999). Therefore, we hypothesized that an upregulation of DAT by DM would enhance cocaine induced hyperlocomotion. Male C57BL/6 retired breeder mice were treated with 6 mg/kg DM i.p. on days 1, 8, and 15. On day 16, half of the treated mice were challenged with cocaine (15 mg/kg) and open field activity was then measured. Animals were sacrificed one day later and the striatum was removed for neurochemical determinations. DM increased habituated locomotor activity (LA) by 400% and enhanced cocaine-induced LA by an additional 58%. DAT expression was increased by about 36% in DM treated animals as measured by both western blotting and [3H] WIN 35,428 binding. Additionally, DM increased tyrosine hydroxylase (TH) by 52%, serine 40 phosphorylated TH by 13% and vesicular monoamine transporter 2 (VMAT2) by 12%, as measured by western blotting. These data suggest that upregulation of DAT by DM enhances the motor excitatory effects of cocaine and may lead to behavioral sensitization. This could have important implications for interactions between the environment and development of drug abuse (Duffard & de Duffard 2002). (supported by DAMD – 00267036).

METHODS

Dosing regimen: Mice were injected i.p with deltamethrin in methoxy triglycol vehicle at 0, 0.1, 1, 3, 6, or 9 mg/kg on days 1, 8, and 15 as described in Kirby et al. (1999).

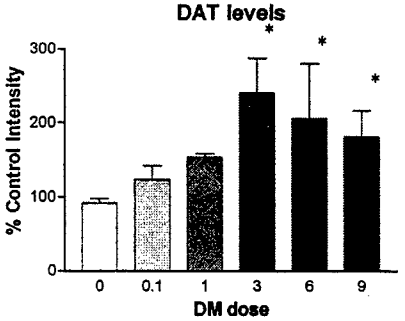
Locomotor activity: Mice were habituated to the test chambers for 1 hour each day for the week preceding the test day. Basal LA was assessed over 1 hour one day after the last DM injection and cocaine induced LA over 1.5 hours one week after the last DM injection.

Binding: Crude synaptosomal preparations were made from left striatum. Synaptosomes were incubated with 25 nM [3H]WIN 35,428 for 2 hours at 0 C. Assay was terminated by rapid filtration through Whatman GF/B filters. Plates were allowed to dry overnight then read on a Packard TopCount.

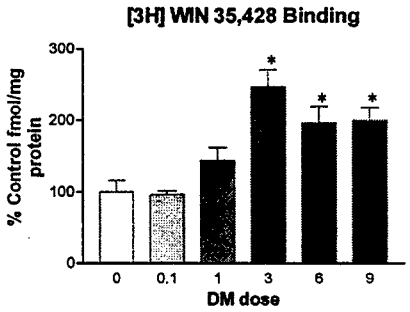
Western Immunoblotting: Western blots were performed as described by Miller et al. (1997) for the dopamine transporter (DAT), tyrosine hydroxylase (TH), and phosphor(Ser40) tyrosine hydroxylase (P-TH). Blots were stripped and alpha tubulin levels were determined to ensure equal protein loading among samples.

HPLC: Dissected striata were sonicated in 0.1 M perchloric acid and centrifuged at 16,000g for 20 min. The supernatants were analyzed for levels of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) by HPLC with an 8-channel coulometric array (ESA).

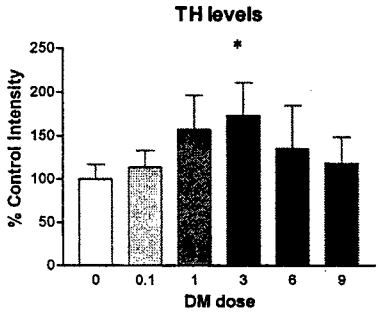
RESULTS



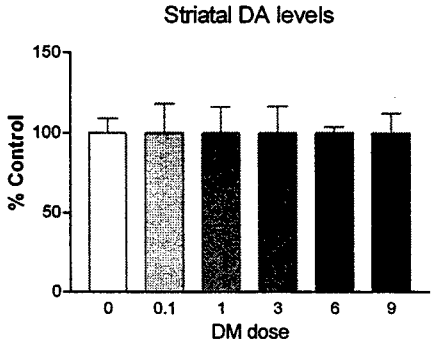
DAT protein is increased at 3 mg/kg *P<0.05



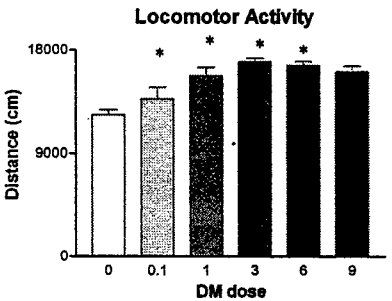
DAT binding is increased by DM *P<0.05



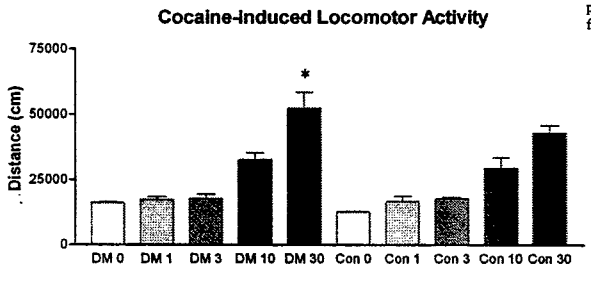
DAT protein is increased at 3 mg/kg *P<0.05



DA levels in the striatum are not affected by DM treatment



Deltamethrin significantly increased basal locomotor activity at several dosages (1,3,6,9 mg/kg) *P<0.05



DM at 3 mg/kg increases the locomotor response to cocaine at 30 mg/kg *P<0.05

DISCUSSION

Here we have demonstrated that deltamethrin increases the levels of DAT and TH in mice The increases in DAT support the findings of Kirby et al. (1999) that show increased uptake of [³H] DA in synaptosomes prepared from DM treated mice.

The dopamine system appears to be preferentially affected by deltamethrin, as no changes were observed in binding experiments for NET or SERT or striatal serotonin levels (data not shown).

Deltamethrin has been shown to increase basal LA in rats (Husain et al. 1996, Brodie & Opacka 1985) similar to that observed here.

DAT is a target for psychostimulants and is necessary for the locomotor activating effects of cocaine, as animals lacking DAT do not respond to cocaine (Uhl et al. 2002).

The differences in cocaine-induced LA when mice are given deltamethrin suggest that the dopaminergic system is more sensitive to cocaine, most likely because of increased DAT levels.

The mechanism for the increase in DAT is unclear. DM produces excitation by interfering with inactivation of voltage-gated sodium channels. This could lead to excess DA release. A greater DA release could lead to upregulation of DAT to maintain synaptic levels of DA.

Another possible mechanism could be an increased production of the transcription factor Nurr1. Nurr1 is responsible for the dopaminergic phenotype of expressing DAT, TH, and VMAT2 (Kim et al.2003). By increasing neuronal activity, Nurr1 levels could go up and lead to greater expression of the aforementioned markers.

By changing DA production and clearance proteins, the homeostasis of the system is disturbed. This can make an organism more sensitive to challenge.

Taken in concert, these data suggest that environmental exposures may lead to an altered response to psychostimulants, which may result in increased risk for abuse .

REFERENCES

Bloomquist et al. (2002). Neurotoxicology 23:537-544
Brodie & Opacka (1985). Arch. Pharm. 331: 341-346
Donovan et al. (1999). Mol. Brain Res. 73:37-49
Duffard & de Duffard (2002). Ann NY Acad. Sci. 965:305-313
Husain et al. (1996). J Tox. Env. Health 48:515-526
Kim et al. (2003). J Neurochem. 85:1443-1454
Miller et al. (1997). Ann. Neurol. 41:530-39
Kirby et al. (1999). Pest. Biochem. Phys. 65:160-168
Uhl et al. (2002). Mol. Psych. 7:21-26

Supported by DAMD - 00267036

Pesticide exposure upregulates the dopamine transporter and increases cocaine-induced locomotor activity while abolishing place preference

Thomas S. Guillot[1] ,Jason R. Richardson[1,2], and Gary W. Miller[1,2]

Center for Neurodegenerative Disease[1] and Department of Environmental and Occupational Health[2], Rollins School of Public Health, Emory University, Atlanta, GA.

ABSTRACT

Deltamethrin (DM), a type II pyrethroid insecticide, has become a widely used in both agricultural and household settings. Pyrethroid exposure has been shown to preferentially affect dopaminergic neurons. A functional upregulation of the dopamine transporter (DAT) has been demonstrated in the striatum of C57BL/6 mice exposed to DM. DAT, in addition to its normal role in dopamine clearance, is a target for psychostimulants such as cocaine. Therefore, we hypothesized that an upregulation of DAT by DM would alter cocaine induced hyperlocomotion and CPP. Male C57BL/6 mice (6 weeks old) were treated with 3 mg/kg DM i.p. or vehicle on days 1, 8, and 15. DAT levels in DM treated animals were doubled as measured by both western blotting and [3H] WIN 35,428 binding. One week after the last dose, vehicle and DM treated mice were challenged with saline or cocaine (30 mg/kg) and locomotor activity was measured. Cocaine increased locomotor activity of controls by 3 fold and DM mice exhibited a 3.5 fold increase in locomotor activity. CPP testing was performed one week after last DM or vehicle injection. Vehicle controls trained with 20 mg/kg cocaine exhibited place preference. However, no place preference was established in DM mice trained with an identical dose of cocaine. These data suggest that upregulation of DAT by DM enhances the motor excitatory effects of cocaine and may lead to behavioral sensitization. The lack of CPP establishment may be due to a deficit in learning or a decrease in the rewarding effect of cocaine. These data demonstrate that DM exposure alters DAT levels and behavioral responses to cocaine. These responses to cocaine may be a result of differential effects of DM on the nigrostriatal and mesolimbic dopamine systems.

INTRODUCTION

Deltamethrin (DM) is a Type II pyrethroid with both agricultural and household uses. The use of this insecticide and other pyrethroids have been steadily increasing since the early 1980s. Other insecticides such as the organochlorines and organophosphates have been banned or phased out over the last three decades.

The main molecular action of pyrethroids is on neuronal sodium channels. These insecticides slow the inactivation of the sodium channel leading to prolonged excitation and irregular discharge of action potentials (Tabarean and Narahashi, 1998). Most interestingly, previous observations have determined that exposure of adult mice to pyrethroids alters the dopaminergic system (Kirby et al., 1999, Gillette and Bloomquist 2003). Specifically, Kirby et al. found the function of the dopamine transporter (DAT) is increased by ~70% after a two week exposure to DM. Many psychostimulants utilize dopamine to elicit their locomotor activating and rewarding effects and DAT is a major target of these drugs.

DAT blockade is a major mechanism of cocaine action (Uhl et al. 2002). However, it also blocks the serotonin (SERT) and norepinephrine (NET) transporters. Genetic knockouts have shown that animals without DAT have locomotor inhibition to cocaine but can still acquire a place preference (Sora et al., 1998, Gainetdinov et al., 1999). However, double knockouts of DAT and SERT do not establish a place preference (Sora et al. 2001). In addition, NET knockouts have increased locomotor activity and reward in response to cocaine (Xu et al., 2000). Little data, however, exists on the responses of dopaminergic challenge when DAT is upregulated exogenously by an environmental contaminant.

METHODS

Animal Treatments:

Male C57BL/6J mice (6 weeks old) were injected i.p. with deltamethrin (99% Chem Service) in methoxy triglycol vehicle in a volume of 10 µL. Injections were given on days 1, 8, & 15.

Dopaminergic Neurochemistry:

For determination of DAT binding sites, striatal membranes were incubated with 25 nM ^{3}H-WIN 35,428 for 2 hours at 0°C in 96-well plates. Non-specific binding was determined by the inclusion of 10 µM GBR 12935.

NET and SERT levels in cortical membranes were determined by the binding of ^{3}H-nisoxetine (5 nM at 0°C for 3 hours, 10 µM desipramine for nonspecific) and ^{3}H-paroxetine (1 nM at 25°C for 1 hour, 10 µM fluoxetine for nonspecific), respectively.

Analysis of dopamine (DA), serotonin (5HT), and norepinephrine (NE) by HPLC was determined as described previously (Richardson and Miller, 2004).

Locomotor Activity:

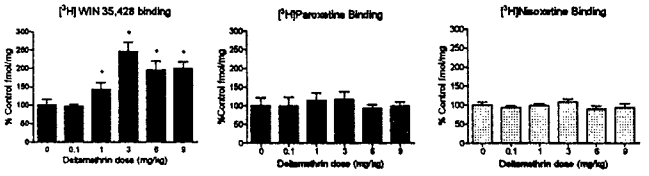
Locomotor activity was observed over 1 hour one day after last injection of deltamethrin. Cocaine-induced locomotor activity was tested 1 week after last deltamethrin injection and monitored for 1 hour.

Conditioned Place Preference:

Conditioning took place on the four days after the last deltamethrin injection. Cocaine (20 mg/kg) was alternated with saline injections four hours apart. Pretesting interval was 10 min and took place one day prior to the final deltamethrin injection. Testing interval was 20 min and was performed one day after last conditioning day. Pretesting, conditioning, and testing utilized a San Diego Instruments three chambered place preference apparatus

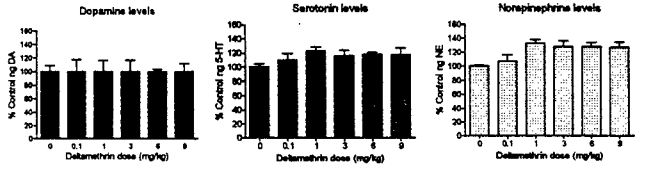
RESULTS

BINDING



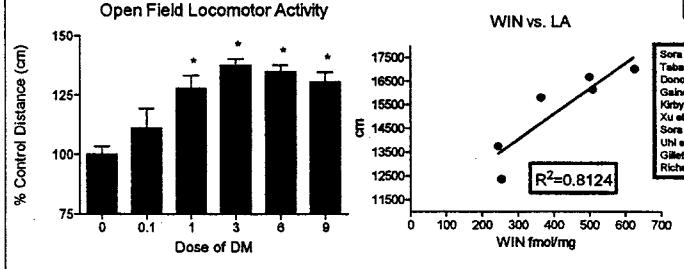
Deltamethrin increased DAT expression in striatum but did not affect SERT or NET levels in the cortex

HPLC

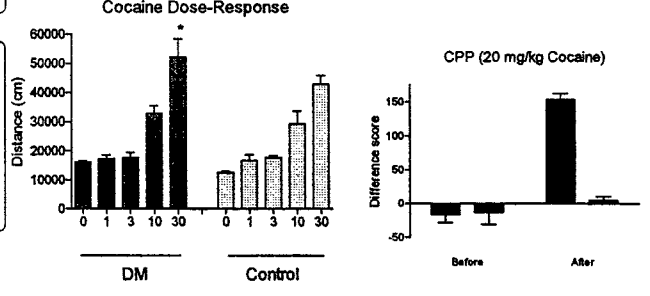


Deltamethrin did not significantly change monoamine levels in the striatum

LOCOMOTOR ACTIVITY



COCAINE

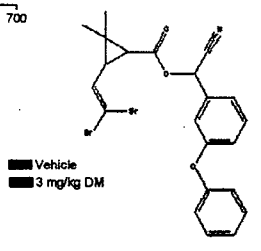


CONCLUSIONS

- Deltamethrin preferentially increases the levels of the dopamine transporter in the striatum. This is in agreement with previous observations using uptake (Kirby et al. 1999)
- There is no significant change in the levels of striatal monoamines
- Open field locomotor activity is increased and this is positively correlated with increases in the dopamine transporter
- Cocaine-induced locomotor activity is increased at a high dose. Uhl et al. found that cocaine-induced locomotor activity was increased in a transgenic mouse that expressed 25-35% more dopamine transporter (Donovan et al. 1999)
- Conditioned place preference is severely impaired in deltamethrin treated animals at a dose that maximally upregulates the dopamine transporter
- It is unclear if these mice have a decreased ability to learn a preference or decreased reward value of cocaine

REFERENCES

Sora et al. *PNAS* 95 (1998) 7699-7704
Tabarean and Narahashi *JPET* 284 (1998) 958-965
Donovan et al. *Mol Brain Res* 73 (1999) 37-49
Gainetdinov et al. *Science* 283 (1999) 397-401
Kirby et al. *Pest Biochem & Phys* 65 (1999) 160-168
Xu et al. *Nat Neurosci* 3 (2000) 465-471
Sora et al. *PNAS* 98 (2001) 5300-5305
Uhl et al. *Mol Psychiatry* 7 (2002) 21-26
Gillette and Bloomquist *Toxicol App Pharm* 192 (2003) 287-293
Richardson and Miller *Toxicol Lett* 148 (2004) 29-40



Deltamethrin

Supported by DAMD 00267036, NIH F32ES013457, and EPA STAR Fellowship #91643701-0

Effects of Pyrethroid Pesticide Exposure on the Dopamine Transporter

Mohamed A. Elwan[1], Jason R. Richardson[1,2], Thomas S. Guillot[1], W. Michael Caudle[1], and Gary W. Miller[1,2]

[1]Center for Neurodegenerative Disease and [2]Department of Environmental and Occupational Health, Rollins School of Public Health, Emory University, Atlanta, GA 30322, USA.

Corresponding Author:

Gary W. Miller, Ph.D.
Center for Neurodegenerative Disease
Whitehead Biomedical Research Building 505M
Emory University
615 Michael Street
Atlanta, GA 30322
USA.
Phone: +404-712-8582
Email: gary.miller@emory.edu

Parkinson's disease (PD) is a progressive neurodegenerative disease affecting the nigrostriatal dopaminergic pathway. Several epidemiological studies have demonstrated an association between pesticide exposure and the incidence of PD. Studies from our laboratory and others have demonstrated that certain pesticides increase levels of the dopamine transporter (DAT), an integral component of dopaminergic neurotransmission and a gateway for dopaminergic neurotoxins. Here, we report that repeated exposure (3 injections over 2 weeks) of mice to two commonly used pyrethroid pesticides, deltamethrin (3 mg/kg) and permethrin (0.8 mg/kg), increases DAT-mediated dopamine uptake by 31 and 28%, respectively. Using cells stably expressing DAT, we determined that exposure (10 min) to deltamethrin and permethrin (1 nM-100 μM) had no effect on DAT-mediated dopamine uptake. Extending exposures to both pesticides for 30 min (10 μM) or 24 hr (1, 5, and 10 μM) resulted in significant decrease in dopamine uptake. This reduction was not the result of competitive inhibition, loss of DAT protein, or cytotoxicity. However, there was an increase in cytoplasmic oligonucleosomes, an index of apoptosis, in cells exhibiting reduced uptake at 24 hr. These data suggest that up-regulation of DAT by *in vivo* pyrethroid exposure is an indirect effect and that longer-term exposure of cells results in apoptosis. Since DAT can greatly affect the vulnerability of dopamine neurons to neurotoxicants, up-regulation of DAT by deltamethrin and permethrin may increase the susceptibility of dopamine neurons to toxic insult, which may provide insight into the association between pesticide exposure and PD.

Key words: Deltamethrin; Permethrin, Pyrethroid, Dopamine transporter; Parkinson's Disease

Parkinson's disease (PD) is a disabling neurodegenerative disorder characterized by the loss of nigrostriatal dopamine neurons and the formation of intraneuronal inclusions termed Lewy bodies (Olanow and Tatton, 1999). Although the exact etiology of PD is unknown, both genetic and environmental factors are thought to contribute to the pathogenesis of PD. While there are rare instances of genetically-linked PD, data from a recent large twin study, found no significant contribution of genetics to late-onset PD (Tanner et al., 1999). This finding suggests that environmental factors or gene-environment interactions play an integral role in the development of sporadic PD.

Several epidemiological studies have identified pesticide exposure as a significant risk factor for Parkinson's disease (Gorell et al., 1998; Le Couteur et al., 1999; Priyadarshi et al., 2000; Tanner and Langston, 1990). Other studies have demonstrated that drinking well-water and living in a rural setting, both of which may increase exposure to agricultural pesticides, increase the risk of developing PD (Barbeau et al., 1987; Betemps and Buncher, 1993; Golbe et al., 1990; Rajput et al., 1986; Rajput et al., 1987; Semchuk et al., 1991). In addition, exposure to pesticides used in the home has been linked to PD (Stephenson, 2000). However, the majority of studies have not identified specific pesticides or the mechanism by which pesticides damage the dopaminergic system and increase the risk of PD.

Studies by our laboratory and others have demonstrated that exposure of mice to the organochlorine insecticide heptachlor increases the expression of the plasma membrane dopamine transporter (DAT; Miller et al., 1999a; Kirby et al., 2001) at dosage levels that elicit no overt toxicity. DAT is an integral component of normal dopamine function and is responsible for terminating dopamine neurotransmission by rapid reuptake of dopamine into the presynaptic terminal (Giros and Caron, 1993; Miller et al., 1999b; Shimada et al., 1991). Several studies have demonstrated that alterations in the expression of DAT can greatly affect the vulnerability of the dopamine neuron to neurotoxins such as MPTP. Gainetdinov and colleagues demonstrated the requirement of DAT for the toxicity of MPTP (Gainetdinov et al., 1997), while Donovan and coworkers (1999) have shown that overexpression of DAT in transgenic mice results in greater loss of dopamine neurons following MPTP exposure. Therefore, exposure to pesticides that increase DAT may increase the susceptibility of dopamine neurons to endogenous neurotoxic dopamine metabolites or exogenous neurotoxicants by increasing their uptake by DAT.

In addition to heptachlor, exposure of mice to the pyrethroid pesticides deltamethrin and permethrin has been demonstrated to increase DAT-mediated dopamine uptake (Gillette and Bloomquist, 2002; Karen et al., 2001; Kirby et al., 1999). Pesticides in the pyrethroid class are widely used in household and agricultural applications and are popular because of their low mammalian toxicity. Although pyrethroids are usually considered environmentally labile, they readily cross the blood brain barrier and can achieve considerable concentrations in the brain (Anadon *et al.*, 1996). Acute toxicity of pyrethroids is primarily mediated through interaction with sodium channels, leading to prolonged depolarization and hyperexcitation of the nervous system (Narahashi, 1982; Tabarean and Narahashi, 2001). Pyrethroids have also been shown to be potent releasers of neurotransmitters, including dopamine (Eels, and Dubocovich, 1988; Kirby et al., 1999). However, the mechanism by which pyrethroids are capable of increasing DAT-mediated dopamine uptake is not clear.

Here, we report that *in vivo* exposure to deltamethrin and permethrin not only causes functional up-regulation of dopamine uptake, but increased levels of DAT as well. In addition, acute exposure of SK-N-MC neuroblastoma cells to these pyrethroids has no effect on dopamine uptake, indicating that deltamethrin and permethrin do not directly interact with DAT. Finally, we found that longer-term exposure to deltamethrin and permethrin reduce dopamine uptake in these cells, and that this effect is most likely the result of an ongoing apoptotic process. Taken together, our results suggest that the effects of pyrethroids on DAT are indirect and that longer-term exposures may be capable of damaging cells through an apoptotic mechanism.

MATERIALS AND METHODS

Materials. Analytical grade (purity $\geq$ 98%) deltamethrin and permethrin were obtained from ChemService Inc. (West Chester, PA). [^{3}H]-dopamine (58 Ci/mmol) and [^{3}H]-WIN 35,428 (85 Ci/mmol) were purchased from Perkin-Elmer Life Sciences (Boston, MA). The rat monoclonal antibody to DAT was purchased from Chemicon (Temecula, CA) and the secondary antibody coupled to horseradish peroxidase was purchased from ICN (Costa Mesa, CA). Super Signal West substrate and stripping buffer were obtained from Pierce (Rockford, IL). All other reagents were obtained from Sigma Chemical Co. (Sigma, St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Animals and Treatments. Male C57BL/6j mice (8 weeks of age) were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were group housed (6 per cage) under a 12:12 light-dark cycle and acclimatized for 1 week prior to initiation of experiments. Standard rodent chow and tap water was available *ad libitum*. All procedures were conducted in accordance with the *Guide for Care and Use of Laboratory Animals* (National Institutes of Health) and previously approved by the Institutional Animal Care and Use Committee at Emory University.

A total of 24 mice were used for these experiments. Control mice were injected intraperitoneally with vehicle (methoxytriglycol; n = 12) and treated mice were injected with deltamethrin (3 mg/kg; n = 6) or permethrin (0.8 mg/kg; n = 6) three times over a 2-week period (Days 1, 8, 15) as described previously (Kirby et al., 1999; Miller et al., 1999b). One day following the last treatment, animals were sacrificed and the striatal tissue dissected out freshly prepared for assay as described below.

Synaptosomal Dopamine Uptake and ^{3}H-WIN 35,428 Binding. Dopamine uptake studies were performed as described previously (Miller et al., 1999b). Briefly, crude synaptosomes were prepared from fresh striatal tissue and incubated in assay buffer (4 mM Tris, 6.25 HEPES, 120 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.6 mM ascorbic acid, 5.5 mM glucose, 10 μM pargyline; pH 7.4) containing a saturating concentration of dopamine (1 μM final concentration) and a tracer amount of [^{3}H]-dopamine (20 nM). A single saturating concentration of dopamine was chosen to assess effects of pyrethroids on the V_{max} of DAT, since previous studies using the same dosing paradigm have demonstrated no significant effect on K_m (Kirby et al., 1999; Karen et al., 2001). Uptake was allowed to proceed for 3 min at 37°C, and then terminated by the addition of ice-cold buffer and rapid vacuum filtration over GF/B filter paper using a Brandel harvester. Filters were washed twice more with buffer, allowed to air dry, and placed in scintillation vials containing 8 mls of Econoscint (Fisher Scientific, Pittsburgh, PA) for scinitillation counting. Uptake rates were calculated as specific uptake (total uptake – non-specific uptake), with non-specific uptake defined by the inclusion of 10 μM nomifensine. Following determination of synaptosomal protein concentration (Bradford, 1976), uptake rates were calculated as pmol/min-mg protein and expressed as percentage of control values.

Determination of ^{3}H-WIN 35,428 binding to DAT was performed essentially as described by Coffey and Reith (1994) with modifications to reduce the total volume to 200 μl, for assay in

96-well microtiter plates. Preliminary kinetic studies indicated that the binding of ^{3}H-WIN 35,428 to striatal synaptosomes was best fit to a one-site model determined by non-linear curve fitting techniques (GraphPad Prism 3.0) with a K_d of 6.58 nM and a B_{max} of 1.08 pmol/mg protein. Therefore, binding studies with crude synaptosomes were conducted with a single concentration (10nM) of ^{3}H-WIN 35,428 in 25 mM sodium phosphate buffer (125 mM NaCl, 5 mM KCl; pH 7.4) for one hour at 4°C in 96-well plates. Incubations were terminated by rapid vacuum filtration onto GF/B filter plates and radioactivity was determined by liquid scintillation counting. Non-specific binding was determined by the inclusion of 10μM nomifensine and specific binding was calculated as the total binding (incubated without 10 μM nomifensine) minus non-specific binding (incubated with nomifensine). Data were calculated as pmol/mg protein and expressed as percentage of control values.

Cell Culture. SK-N-MC (human neuroblastoma) cells stably expressing human DAT (SK-DAT; Stephans et al., 2002) were maintained in minimum essential medium (MEM) supplemented with Earle's salts, 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and non-essential amino acids, and incubated under a humidified atmosphere of 5% CO_2 in air at 37°C. For pyrethroid exposure, deltamethrin and permethrin were dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 mM. Further dilutions of both pyrethroids were made in uptake buffer for experiments with dopamine uptake and in serum-free media for experiments with cytotoxicity and apoptosis. The final concentration of DMSO was ≤0.1% for all experiments. Control experiments were performed in the presence of DMSO in a concentration similar to that used in the pyrethroid-treated cells.

Dopamine Uptake and Western Blot Studies in Cells. Dopamine uptake by SK-DAT cells was performed as described elsewhere (Pifl et al., 1993). Briefly, cells were plated in 24-well plates and incubated for 48 hrs in the above MEM medium. Cells were washed once with the uptake buffer (4 mM Tris, 6.25 HEPES, 120 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.6 mM ascorbic acid, 5.5 mM glucose; pH 7.4). For acute studies (10 and 30 min), cells were incubated with various concentrations of either deltamethrin or permethrin (100 nm-10 μM). For longer term studies (24 hr), cells were exposed to the pyrethroids in serum-free

media for 24 hr and then washed once in uptake buffer. Following the wash step, cells were incubated for 5 min at 37°C with uptake buffer containing unlabeled DA (2.5 μM) and a tracer amounts of [^{3}H]-dopamine. Pargyline (10 μM) was included during all the uptake periods to inhibit monoamine oxidase and non-specific uptake was defined in the presence of 10 μM GBR-12935. After the incubation period, the buffer was quickly aspirated off and cells were washed twice with ice-cold buffer. Cells were then dissolved in 0.5 ml of 0.1 M NaOH and the solubilized cellular contents were transferred to liquid scintillation vials containing 8 ml of liquid scintillation cocktail. The radioactivity was measured by scintillation counting and an aliquot of the solubilized cells was used for protein determination using bovine serum albumin as standard (Lowry *et al.*, 1951). Uptake rates were calculated as specific uptake (total uptake – non-specific uptake) and expressed as percentage of control values.

To determine the effects of pyrethroids on the K_m and V_{max} of dopamine uptake in SK-DAT cells, cells were incubated with pyrethroids for 10 min or 24 hr and dopamine uptake was determined as described above using increasing concentrations (0.5-40 μM) of dopamine. K_m and V_{max} were determined by non-linear regression using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA).

Western blots were performed as previously described (Miller et al., 1997). Briefly, cells were scraped from culture plates and sonicated at 4°C in a buffer containing 300 mM sucrose, 10 mM HEPES and 1 μg/ml of leupeptin, aprotinin, and pepstatin. Samples (20 μg) were subjected to SDS PAGE on 10% precast NuPage gels (InVitrogen, Carlsbad, CA). Samples were electrophoretically transferred to a polyvinylidene difluoride membrane, and nonspecific sites were blocked in 7.5% nonfat dry milk in Tris-buffered saline (135 mM NaCl, 2.5 mM KCl, 50 mM Tris, and 0.1% Tween 20, pH 7.4). Membranes were then incubated in a monoclonal antibody (Chemicon, Temecula, CA) to the N-terminus of DAT (Miller et al., 1997). Antibody binding was detected using a goat anti-rabbit horseradish peroxidase secondary antibody (ICN, Costa Mesa, CA) and enhanced chemiluminescence. The chemiluminescent signal was captured on an Alpha Innotech Fluorchem imaging system and stored as a digital image. Densitometric analysis was performed and calibrated to co-blotted dilutional standards of pooled cells from all control samples. Membranes were then stripped for 15 min at 25°C with Pierce Stripping Buffer and reprobed with a monoclonal α-tubulin antibody to ensure equal protein loading across samples.

Cytotoxicity and DNA Fragmentation Assays. The possible cytotoxic effects of pyrethroid exposure on SK-DAT cells was evaluated by measuring lactate dehydrogenase (LDH) leakage into the extracellular fluid using a cytotoxicity detection kit (Roche Applied Science, Indianapolis, IN). Briefly, cells ($1x10^4$ cells/well) were incubated with different concentrations of pyrethroids for 24 hr in serum-free MEM and the incubation medium was collected and centrifuged. The cell-free supernatant (100 µl) was then mixed with 100 µl of the catalyst-dye mix (included in kit) in a 96-well microtiter plate. LDH activity in the media was determined spectrophotometrically at 490 nm by monitoring the increase in absorbance over a 30 min period. To determine the total amount of LDH in each sample, the original cells and media were lysed in 1% Triton X-100 for 30 min and LDH activity was determined as described above. The LDH release for each sample was defined as the LDH activity in the incubation media divided by the total amount of LDH activity following Triton-lysis and data expressed presented as percentage LDH leakage.

The possible apoptotic effect of pyrethroid exposure on SK-DAT cells was evaluated by DNA fragmentation assay using the Cell Death Detection ELISA Plus Assay kit (Roche Applied Science, Indianapolis, IN). This kit measures amount of histone-associated low molecular weight DNA, which is indicative of histone-associated DNA fragments which have been cleaved by endonuclease, in the cytoplasm of cells and has been used as a measure of apoptosis in cells exposed to other toxicants (Anantharam et al., 2002; Kitazawa et al. 2002). Briefly, cells were seeded in microplate wells ($1x10^4$ cells/well) and treated for 24 hr in serum-free MEM with either deltamethrin or permethrin. After treatment, cells were pelleted and washed once with phosphate-buffered saline. Cells were then incubated with lysis buffer (supplied with the kit) at room temperature for 30 min and centrifuged. Aliquots of supernatant (20 µl) were dispensed into a streptavidin-coated 96-well microtiter plate (supplied with the kit) and incubated with 80 µl of antibody cocktail for 2 hr at room temperature with shaking. The antibody cocktail consisted of a mixture of anti-histone biotin and anti-DNA-HRP, which binds to both single-stranded DNA and double-stranded DNA, which are major constituents of nucleosomes. After incubation, plates were washed with incubation buffer and determination of the amount of nucleosomes retained by anti-DNA-HRP was determined spectrophotometrically with 2,2'-azino-di[3-ethoxybenzyl thiazoline sulfonate] as an HRP substrate (supplied with the kit).

Measurements were made at 405 nm using a Spectramax Plus microplate reader (Molecular Devices). Non-specific signal was determined by subtraction of a reagent blank and data were expressed as mU (defined as absorbance x 10^{-3}) cytoplasmic oligonucleosomes.

Statistical analysis. Results were expressed as the mean ± S.E.M. In instances where data were presented as percentage of control, all statistical procedures were performed on the raw numbers. Data were analyzed by Student's *t*-test or one way analysis of variance (ANOVA). If a significant F was determined by ANOVA, post-hoc analysis was performed with Dunnett's test. Statistical significance is reported at the $p \leq 0.05$ level.

RESULTS

No overt signs of toxicity, defined as tremor, choreoathetosis, and salivation, were observed following administration of either deltamethrin or permethrin. There were also no significant changes in weight in any of the treated animals (data not shown).

Administration of deltamethrin (3 mg/kg) and permethrin (0.8 mg/kg) three times over two weeks resulted in a significant increase in striatal dopamine uptake (Fig. 1A). Deltamethrin exposure increased DAT-mediated dopamine uptake in striatal synaptosomes by 31% ($p < 0.01$) one day following the last treatment. At this same time, permethrin exposure increased dopamine uptake by 28% ($p < 0.01$). The increases in dopamine uptake observed were accompanied by increases in DAT-binding sites as determined by ^{3}H-WIN 35,428 binding in striatal synaptosomes (Fig. 1B). Deltamethrin resulted in a 32% increase ($p < 0.01$), while permethrin exposure increased DAT-binding sites by 24% ($p < 0.01$).

Since we observed significant up-regulation of DAT following *in vivo* exposure to deltamethrin and permethrin, we sought to determine whether these effects were the result of direct action of the pyrethroids on DAT. To accomplish this, we exposed SK-N-MC neuroblastoma cells stably expressing DAT (SK-DAT) to various concentrations of pyrethroids for 10 min, 30 min, or 24 hr. Exposure of SK-DAT cells for 10 min with either deltamethrin or permethrin (1 μM to 10 μM) had no significant effect on DAT-mediated dopamine uptake (Fig. 2A). Extending the incubation time to 30 min resulted in a significant decrease in dopamine uptake by both deltamethrin (20%; $p < 0.01$) and permethrin (18%; $p < 0.01$) only at a concentration of 10 μM (Fig. 2B). Further extending the incubation time to 24 hr resulted in a

greater decrease of dopamine uptake, as both deltamethrin ($p < 0.01$) and permethrin ($p < 0.01$) decreased dopamine uptake by about 75% at concentrations of 5 and 10 μM (Fig. 2C). To determine the nature of the inhibition of DAT-mediated uptake by deltamethrin and permethrin, we performed kinetic analysis of dopamine uptake in SK-DAT cells exposed to 10 μM of either compound for 24 hr. Both pyrethroids showed significant alterations in V_{max}, with little effect on K_m, suggesting that the decreased uptake may be the result of non-competetive inhibition (Fig. 3A and B). Similar results were observed following 30 min incubations (data not shown).

Based upon the time and concentrations required for deltamethrin and permethrin to cause decreased dopamine uptake, we considered that the decreased uptake may be the result of loss of DAT protein. Exposure of cells to 10 μM of deltamethrin or permethrin was without effect on the total levels of DAT as determined by western immunoblotting (Fig. 3C). We next examined whether exposure to deltamethrin or permethrin resulted in cytotoxicity by assessing LDH leakage from the cells into the incubation medium. Treatment of SK-DAT cells with 1 to 10 μM of deltamethrin or permethrin for 24 hr did not produce any significant change in LDH leakage, effectively ruling out overt cytotoxicity as a mechanism for the decreased dopamine uptake. However, exposure of SK-DAT cells to 5 or 10 μM of permethrin or 10 μM deltamethrin for 24 hr significantly increased DNA-fragmentation, an indication of an active apoptotic process (Fig. 4B). Exposure to 5 μM permethrin increased the amount of fragmentation by 191% ($p < 0.05$) and exposure to 10 μM increased fragmentation by 422% ($p < 0.01$). Deltamethrin increased fragmentation by 223% ($p < 0.05$) only at 10 μM. Similarly, increased DNA-fragmentation was observed following 30 min of exposure to 10 μM of deltamethrin (35%; $p < 0.05$) or permethrin (65%; $p < 0.05$). No significant effects were observed with lower concentrations (data not shown).

DISCUSSION

Previous studies have demonstrated that repeated exposure of mice to the pyrethroid pesticides, deltamethrin and permethrin, results in increased synaptosomal dopamine uptake (Gillette and Bloomquist, 2003; Karen et al., 2001; Kirby et al., 1999) In this study, we confirm these observations and extend them by demonstrating that the functional up-regulation is accompanied by increases in DAT-binding sites. In addition, we demonstrate that permethrin and deltamethrin have no direct effect on DAT and that longer-term *in vitro* exposure of cells stably

expressing DAT results in decreased DAT-mediated dopamine uptake and induction of apoptosis.

Deltamethrin and permethrin are members of the pyrethroid class of pesticides which are synthetic derivatives of the naturally occurring pyrethrum from chrysanthemum flowers. These compounds exert their toxicity primarily through binding to sodium channels and prolonging the opening of the channel (Narahashi, 1996). However, recent data suggest that these compounds may specifically target the dopaminergic system. It has been demonstrated that exposure of mice to deltamethrin or permethrin results in an increase in dopamine uptake in striatal synaptosomes, possibly indicative of an up-regulation of DAT (Karen et al., 2001; Kirby et al., 1999). In addition, up-regulation of dopamine uptake following deltamethrin exposure was accompanied by increased binding of GBR 12935 (Gillette and Bloomquist, 2003). In this study, we found significant increases in DAT binding sites as measured with WIN 35,428 that mirrored the increase in DAT-mediated dopamine uptake. While no specific mechanism has been identified for the increase of DAT by these compounds, chemicals known to cause dopamine release, like amantidine, can increase DAT expression (Gordon et al., 1996). If this were to be sustained over time, one would expect that the elevated extracellular dopamine would increase the expression of the dopamine transporter in an attempt to clear and recycle dopamine. Another possibility is up-regulation of DAT at the transcriptional level. The transcription factor Nurr1 is critical for the development of the dopaminergic phenotoype (Zetterstrom et al., 1997) and has been shown to directly enhance transcription of DAT (Sacchetti et al., 2001; Hermanson et al., 2003). Since Nurr1 transcription is enhanced by neuronal activity and membrane depolarization (Perrone-Cappano et al., 2000), dopamine release and/or blockade of sodium channels by deltamethrin may cause upregulation of Nurr1, ultimately leading to increased expression of DAT.

In contrast to the *in vivo* data, our results show that incubation of SK-DAT cells with DM or PM resulted in significant decrease in DA uptake and alteration in the DA uptake kinetics. It is apparent that this decrease in DA uptake is dependent on both the concentration of pyrethroids and the time of incubation. The lack of a significant effect on dopamine uptake after 10 min incubation suggest that both compounds are devoid of any direct effect (increase or decrease) on dopamine uptake. It also rules out a cocaine-like effect on dopamine uptake. However, deltamethrin and permethrin concentrations as low as 1 nM produced slight, but not statistically significant, increase in dopamine uptake by SK-DAT cells (data not shown). This is

consistent with reports that deltamethrin attenuated basal- and quinpirole-potentiated dopamine uptake in rat striatal synaptosomes (Thompson et al., 2000), and that permethrin reduces the level of DAT immunoreactive proteins in rat striatum (Pittman et al., 2003). Our findings in the cell culture experiments are not in agreement with the data showing that pyrethroids increase both striatal DAT protein and maximal DA uptake (Bloomquist et al., 2002; Gillette and Bloomquist, 2003; Karen et al., 2001). It is worth noting, that in these studies the increases in DAT protein and function were observed using small doses of pyrethroids. However, exposure to higher doses of pyrethroids resulted in decreases in DAT level and dopamine uptake possibly due to cell stress and decreased mitochondrial function (Bloomquist et al., 2002; Gillette and Bloomquist, 2003; Karen et al., 2001). Despite the difficulty in comparing pyrethroid concentrations in our experiments with those achieved with *in vivo* studies, it seems that the concentrations we used are in the high range.

Since a direct (cocaine-like) action on DAT is excluded by the results of 10 min experiments, other mechanism(s) of may be involved in the reduction of dopamine uptake observed here. One such possibility is that by interference with the nerve membrane sodium channels, pyrethroids lead to prolonged depolarization (Narahashi, 1982; Tabarean and Narahashi, 2001). Indeed, veratridine, a sodium channel activator, at concentrations of 10 μM and 50 μM resulted in tetrodotoxin-sensitive inhibition of DA uptake into rat striatal synaptosomes (Holz and Coyle, 1974). Another potential mechanism we considered was that the results we observed were because of pyrethroid-induced cytotoxicity. Results of the current study indicate that this is not a likely possibility, however, since at the highest concentration attainable (10 μM), neither deltamethrin nor permethrin produced any significant cytotoxic effect as revealed by LDH assay. This is in agreement with finding that exposure to pyrethroids for 24 h did not produce any significant effect on LDH release from mouse cerebellar granule cells (Imamura et al., 2000), and also indicates that decreased DA uptake observed in our study is not due to cytotoxicity.

In order to specify the deleterious effects of pyrethroids on SK-DAT cells survival, we tested the possibility of apoptotic cell death. Our findings reveal that pyrethroids induce apoptosis following 24 hr incubation with deltamethrin or permethrin (10 μM), whereas at lower concentrations, only permethrin induced apoptosis. These data suggest that apoptosis may explain, in part, some of the observed decrease in dopamine uptake. There is growing body of

evidence indicating that apoptosis might play a crucial role in the toxic actions of pyrethroids by induction of apoptosis and altering the expression of p53, Bax, and Bcl-2 genes (El-Gohary et al., 1999; Wu et al., 2003; Wu and Liu, 2000b; Wu and Liu, 2000a). Also, deltamethrin elicits prolonged expression of c-Fos and c-Jun in the rat cerebral cortex, suggesting that deltamethrin can initiate the neurodegeneration process (Wu and Liu, 2003). Moreover, pyrethroids decrease the viability and arrest mitosis in cultures of V79 Chinese hamster lung cells (Hadnagy *et al.*, 1999), human lymphocytes (Carbonell et al., 1989), and liver cells (el-Tawil and Abdel-Rahman, 1997).

In conclusion, the present study clearly demonstrates that deltamethrin and permethrin increase DAT and DAT-mediated dopamine uptake in striatal synaptosomes following in vivo exposure. However, exposure to these compounds *in vitro* decrease dopamine uptake in SK-DAT cells. Our results also suggest that induction of apoptosis may partly explain some of these findings. These results may shed light the mechanisms underlying pyrethroids-induced neurotoxicity and might implicate pyrethroids as environmental risk factors leading to the development of PD.

ACKNOWLEDGEMENTS

This work was supported by the US Army Medical Research and Materiel Command under Award No. 00267036 (GWM), and in part by NIH F32ES013457 (JRR), and an EPA STAR Fellowship (TSG). Opinions, interpretations, conclusions and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army, NIH, or EPA.

REFERENCES

Anadon, A., Martinez-Larranaga, M. R., Fernandez-Cruz, M. L., Diaz, M. J., Fernandez, M. C., Martinez, M. A., 1996. Toxicokinetics of deltamethrin and its 4'-HO-metabolite in the rat. Toxicol. Appl. Pharmacol. 141, 8-16.

Betarbet, R., Sherer, T. B., MacKenzie, G., Garcia-Osuna, M., Panov, A. V., Greenamyre, J. T., 2000. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat. Neurosci. 3, 1301-1306.

Bickmeyer, U., Weinsberg, F., Wiegand, H., 1994. Effects of deltamethrin on catecholamine secretion of bovine chromaffin cells. Arch. Toxicol. 68, 532-534.

Bloomquist, J. R., Barlow, R. L., Gillette, J. S., Li, W., Kirby, M. L., 2002. Selective effects of insecticides on nigrostriatal dopaminergic nerve pathways. Neurotoxicology 23, 537-544.

Bougria, M., Vitorica, J., Cano, J., Machado, A., 1995. Implication of dopamine transporter system on 1-methyl-4-phenylpyridinium and rotenone effect in striatal synaptosomes. Eur. J. Pharmacol. 291, 407-415.

Carbonell, E., Puig, M., Xamena, N., Creus, A., Marcos, R., 1989. Mitotic arrest induced by fenvalerate in human lymphocyte cultures. Toxicol. Lett. 48, 45-48.

Clark, J. M., Brooks, M. W., 1989. Role of ion channels and intraterminal calcium homeostasis in the action of deltamethrin at presynaptic nerve terminals. Biochem. Pharmacol. 38, 2233-2245.

Doherty, J. D., Morii, N., Hiromori, T., Ohnishi, J., 1988. Pyrethroids and the striatal dopaminergic system in vivo. Comp. Biochem. Physiol. C 91, 371-375.

Eells, J. T., Bandettini, P. A., Holman, P. A., Propp, J. M., 1992. Pyrethroid insecticide-induced alterations in mammalian synaptic membrane potential. J. Pharmacol. Exp. Ther. 262, 1173-1181.

Eells, J. T., Dubocovich, M. L., 1988. Pyrethroid insecticides evoke neurotransmitter release from rabbit striatal slices. J. Pharmacol. Exp. Ther. 246, 514-521.

El-Gohary, M., Awara, W. M., Nassar, S., Hawas, S., 1999. Deltamethrin-induced testicular apoptosis in rats: the protective effect of nitric oxide synthase inhibitor. Toxicology 132, 1-8.

el-Tawil, O. S., Abdel-Rahman, M. S., 1997. Effect of cypermethrin on isolated male and female rat hepatocytes. J. Toxicol. Environ. Health 52, 461-474.

Forshaw, P. J., Lister, T., Ray, D. E., 2000. The role of voltage-gated chloride channels in type II pyrethroid insecticide poisoning. Toxicol. Appl. Pharmacol. 163, 1-8.

Gassner, B., Wuthrich, A., Scholtysik, G., Solioz, M., 1997. The pyrethroids permethrin and cyhalothrin are potent inhibitors of the mitochondrial complex I. J. Pharmacol. Exp. Ther. 281, 855-860.

Gillette, J. S., Bloomquist, J. R., 2003. Differential up-regulation of striatal dopamine transporter and alpha-synuclein by the pyrethroid insecticide permethrin. Toxicol. Appl. Pharmacol. 192, 287-293.

Gorell, J. M., Johnson, C. C., Rybicki, B. A., Peterson, E. L., Richardson, R. J., 1998. The risk of Parkinson's disease with exposure to pesticides, farming, well water, and rural living. Neurology 50, 1346-1350.

Grosse, G., Thiele, T., Heuckendorf, E., Schopp, E., Merder, S., Pickert, G., Ahnert-Hilger, G., 2002. Deltamethrin differentially affects neuronal subtypes in hippocampal primary culture. Neuroscience 112, 233-241.

Hadnagy, W., Seemayer, N. H., Kuhn, K. H., Leng, G., Idel, H., 1999. Induction of mitotic cell division distrubances and mitotic arrest by pyrethroids in V79 cell cultures. Toxicol. Lett. 107, 81-87.

Hartley, A., Stone, J. M., Heron, C., Cooper, J. M., Schapira, A. H., 1994. Complex I inhibitors induce dose-dependent apoptosis in PC12 cells: relevance to Parkinson's disease. J. Neurochem. 63, 1987-1990.

Holz, R. W., Coyle, J. T., 1974. The effect of various salts, temperature, and the alkaloids veratridine and batrachotoxin on the uptake of [3H]dopamine into synaptosomes from rat striatum. Mol. Pharmacol. 10, 746-758.

Hudson, P. M., Tilson, H. A., Chen, P. H., Hong, J. S., 1986. Neurobehavioral effects of permethrin are associated with alterations in regional levels of biogenic amine metabolites and amino acid neurotransmitters. Neurotoxicology 7, 143-153.

Husain, R., Malaviya, M., Seth, P. K., 1994. Effect of deltamethrin on regional brain polyamines and behaviour in young rats. Pharmacol. Toxicol. 74, 211-215.

Imamura, L., Hasegawa, H., Kurashina, K., Hamanishi, A., Tabuchi, A., Tsuda, M., 2000. Repression of activity-dependent c-fos and brain-derived neurotrophic factor mRNA expression by pyrethroid insecticides accompanying a decrease in Ca(2+) influx into neurons. J. Pharmacol. Exp. Ther. 295, 1175-1182.

Kadous, A., Matsumura, F., Enan, E., 1994. High affinity binding of 3H-verapamil to rat brain synaptic membrane is antagonized by pyrethroid insecticides. J. Environ. Sci. Health B 29, 855-871.

Karen, D. J., Li, W., Harp, P. R., Gillette, J. S., Bloomquis, J. R., 2001. Striatal dopaminergic pathways as a target for the insecticides permethrin and chlorpyrifos. Neurotoxicology 22, 811-817.

Lazarini, C. A., Florio, J. C., Lemonica, I. P., Bernardi, M. M., 2001. Effects of prenatal exposure to deltamethrin on forced swimming behavior, motor activity, and striatal dopamine levels in male and female rats. Neurotoxicol. Teratol. 23, 665-673.

Le Couteur, D. G., McLean, A. J., Taylor, M. C., Woodham, B. L., Board, P. G., 1999. Pesticides and Parkinson's disease. Biomed. Pharmacother. 53, 122-130.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., 1951. Protein measurement with Folin-phenol reagent. J. Biol. Chem. 193, 265-275.

Miller, G. W., Kirby, M. L., Levey, A. I., Bloomquist, J. R., 1999. Heptachlor alters expression and function of dopamine transporters. Neurotoxicology 20, 631-637.

Narahashi, T., 1982. Cellular and molecular mechanisms of action of insecticides: neurophysiological approach. Neurobehav. Toxicol. Teratol. 4, 753-758.

Parker, C. M., Albert, J. R., Van Gelder, G. A., Patterson, D. R., Taylor, J. L., 1985. Neuropharmacologic and neuropathologic effect of fenvalerate in mice and rats. Fundam. Appl. Toxicol. 5, 278-286.

Pifl, C., Giros, B., Caron, M. G., 1996. The dopamine transporter. The cloned target site of parkinsonism-inducing toxins and of drugs of abuse. Adv. Neurol. 69, 235-238.

Pittman, J. T., Dodd, C. A., Klein, B. G., 2003. Immunohistochemical changes in the mouse striatum induced by the pyrethroid insecticide permethrin. Int. J. Toxicol. 22, 359-370.

Priyadarshi, A., Khuder, S. A., Schaub, E. A., Priyadarshi, S. S., 2001. Environmental risk factors and Parkinson's disease: a metaanalysis. Environ. Res. 86, 122-127.

Purkerson-Parker, S., McDaniel, K. L., Moser, V. C., 2001. Dopamine transporter binding in the rat striatum is increased by gestational, perinatal, and adolescent exposure to heptachlor. Toxicol. Sci. 64, 216-223.

Ray, D. E., Forshaw, P. J., 2000. Pyrethroid insecticides: poisoning syndromes, synergies, and therapy. J. Toxicol. Clin. Toxicol. 38, 95-101.

Richardson, J. R., Miller, G. W., 2004. Acute exposure to aroclor 1016 or 1260 differentially affects dopamine transporter and vesicular monoamine transporter 2 levels. Toxicol. Lett. 148, 29-40.

Soderlund, D. M., Clark, J. M., Sheets, L. P., Mullin, L. S., Piccirillo, V. J., Sargent, D., Stevens, J. T., Weiner, M. L., 2002. Mechanisms of pyrethroid neurotoxicity: implications for cumulative risk assessment. Toxicology 171, 3-59.

Stephans, S. E., Miller, G. W., Levey, A. I., Greenamyre, J. T., 2002. Acute mitochondrial and chronic toxicological effects of 1-methyl-4-phenylpyridinium in human neuroblastoma cells. Neurotoxicology 23, 569-580.

Tabarean, I. V., Narahashi, T., 2001. Kinetics of modulation of tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels by tetramethrin and deltamethrin. J. Pharmacol. Exp. Ther. 299, 988-997.

Tanner, C. M., Chen, B., Wang, W., Peng, M., Liu, Z., Liang, X., Kao, L. C., Gilley, D. W., Goetz, C. G., Schoenberg, B. S., 1989. Environmental factors and Parkinson's disease: a case-control study in China. Neurology 39, 660-664.

Thompson, T. L., Moore, C. C., Smith, B., 2000. Estrogen priming modulates autoreceptor-mediated potentiation of dopamine uptake. Eur. J. Pharmacol. 401, 357-363.

Wu, A., Li, L., Liu, Y., 2003. Deltamethrin induces apoptotic cell death in cultured cerebral cortical neurons. Toxicol. Appl. Pharmacol. 187, 50-57.

Wu, A., Liu, Y., 2000a. Apoptotic cell death in rat brain following deltamethrin treatment. Neurosci. Lett. 279, 85-88.

Wu, A., Liu, Y., 2000b. Deltamethrin induces delayed apoptosis and altered expression of p53 and bax in rat brain. Environ. Toxicol. Pharmacol. 8, 183-189.

Wu, A., Liu, Y., 2003. Prolonged expression of c-Fos and c-Jun in the cerebral cortex of rats after deltamethrin treatment. Brain Res. Mol. Brain Res. 110, 147-151.

Figure 1. Repeated administration of deltamethrin (DM; 3 mg/kg) or permethrin (PM; 0.8 mg/kg) to C57 mice increases (A) dopamine uptake in striatal synaptosomes and (B) DAT levels in striatal synaptosomes as determined by 3H-WIN 35,428 binding. Data are presented as percentage of control values and represent mean ± SEM (n = 5-6 animals per treatment for pyrethroids and 12 animals for control). * indicates groups are significantly different from control values ($p \leq 0.01$) using the untransformed data as determined by ANOVA followed by Dunnett's test.

Figure 2. Effects of deltamethrin (DM) and permethrin (PM) on dopamine uptake in SK-N-MC neuroblastoma cells stably expressing the human DAT. Cells were incubated with various concentrations of DM or PM for 10 min (A), 30 min (B), or 24 hr (C) and dopamine uptake was determined as described in Materials and Methods. Data are presented as percentage of control values and represent mean ± S.E.M. (n = 3). * indicates groups are significantly different from control values ($p \leq 0.01$) using the untransformed data as determined by ANOVA followed by Dunnett's test.

Figure 3. Effects of (A) deltamethrin (DM; 10 μM) or (B) permethrin (PM; 10 μM) treatment for 24 hr on the kinetics of dopamine uptake in SK-N-MC neuroblastoma cells stably expressing the human DAT. Cells were incubated with DM or PM for 24 hr and the kinetics of dopamine uptake were determined by using varying concentrations of dopamine as described in Materials and Methods. Data represent mean ± S.E.M. (n = 3) and absence of error bars indicates that the standard error resdes within the size of the symbol. (C) Total DAT levels in cells treated with DM or PM for 24 hr as determined by western immunoblotting. Data represent mean ± S.E.M. (n = 3).

Figure 4. Effects of deltamethrin or permethrin on (A) LDH leakage and (B) DNA fragmentation. SK-N-MC cells stably expressing the human DAT were treated with media (C), vehicle (DMSO), deltamethrin (DM; 1-10 μM), or permethrin (PM; 1-10 μM) for 24 hr. After exposure, cell-free media samples were collected

and assayed for LDH levels by spectrophotometry. DNA-fragmentation in cells following 24 hr of exposure was determined as described in Materials and Methods. Data for LDH are presented as percentage of LDH leakage and represent mean ± S.E.M. (n = 3). * indicates groups are significantly different from control values ($p \leq 0.05$) using the untransformed data as determined by ANOVA followed by Dunnett's test.

Figure 1. Elwan et al., 2004

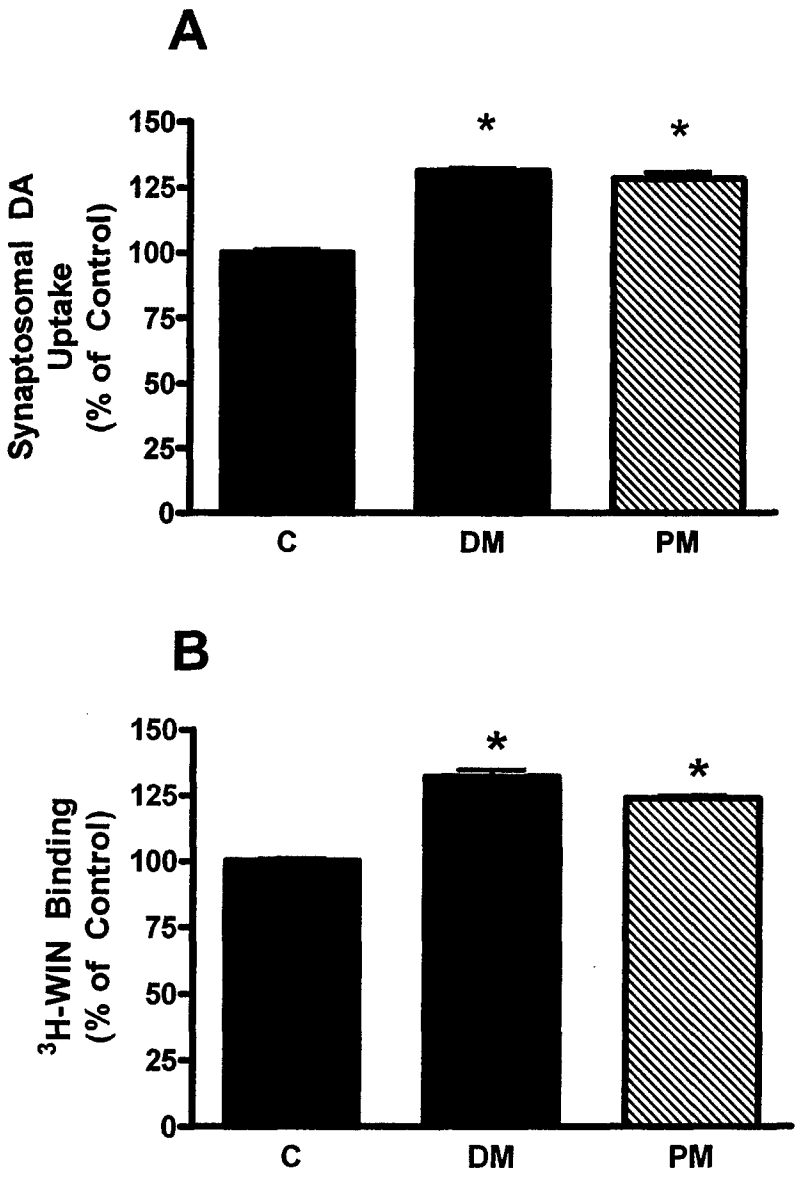


Figure 2. Elwan et al., 2004

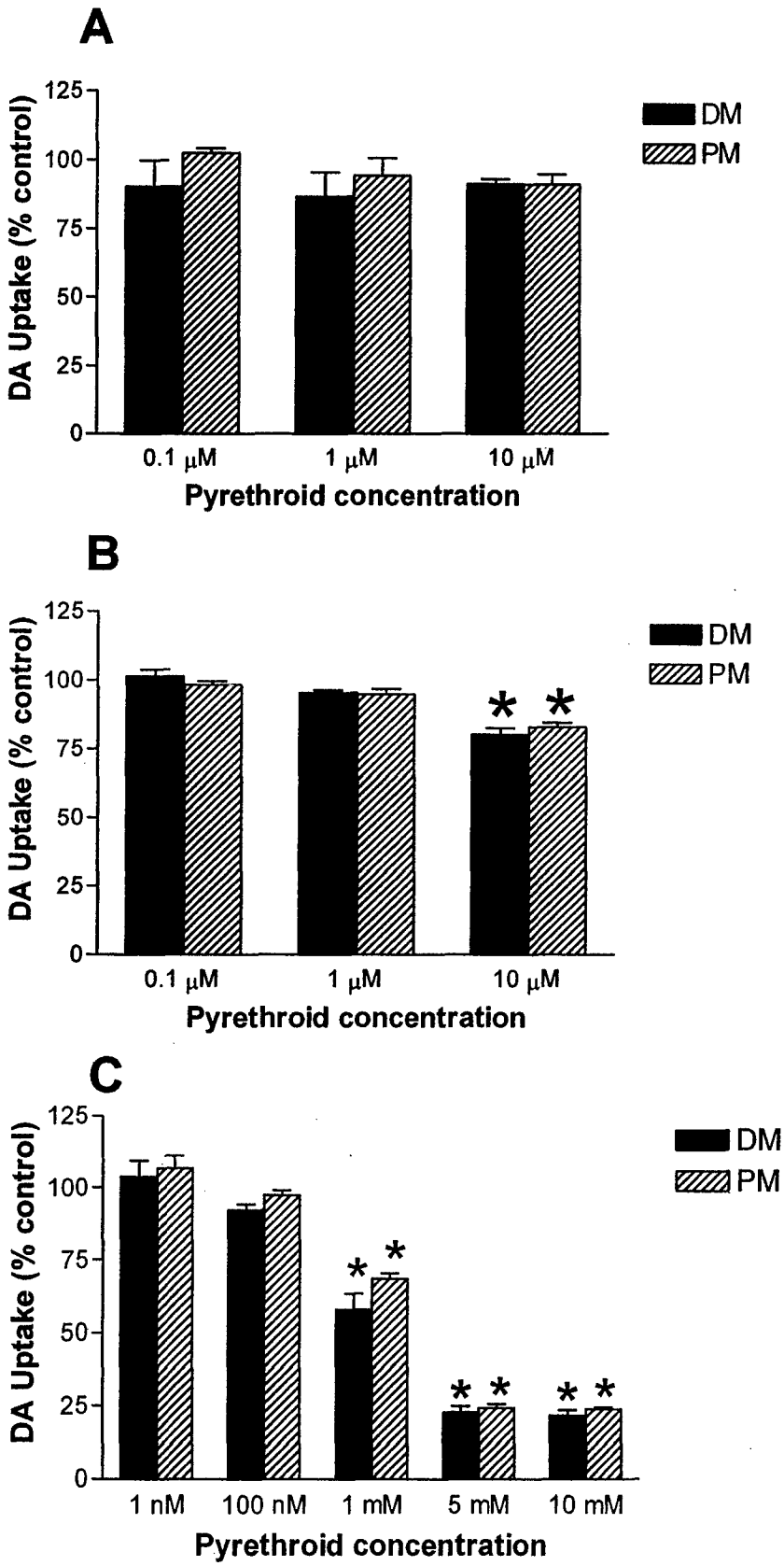


Figure 3. Elwan et al., 2004

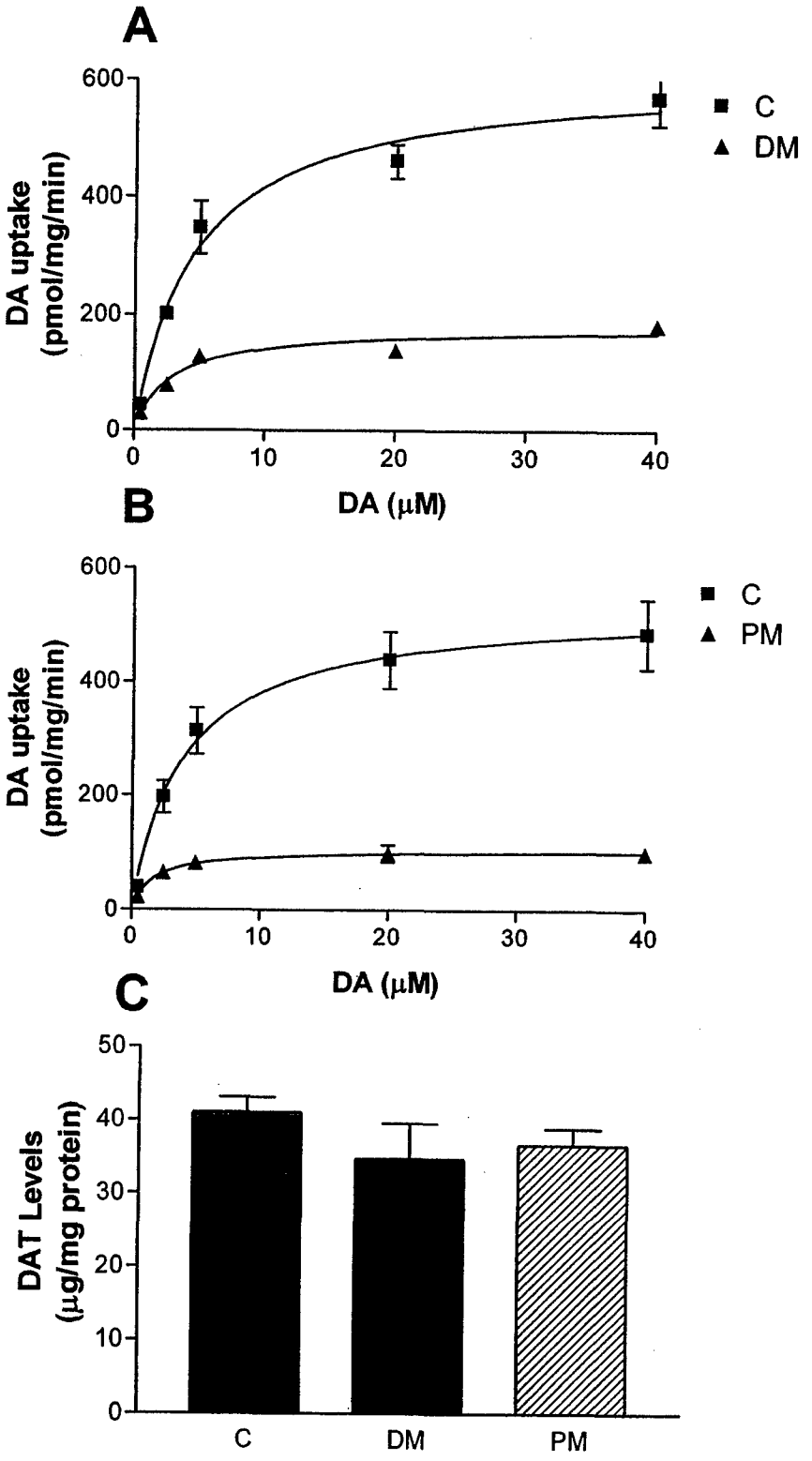


Figure 4. Elwan et al., 2004